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Modulation of Antigen Expression in B-Cell Precursor Acute Lymphoblastic Leukemia During Induction Therapy is Partly Transient: Evidence for a Drug-Induced Regulatory Phenomenon. Results of the AIEOP-BFM-ALL-FLOW-MRD-Study Group

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Background: Changes of antigen expression on residual blast cells of acute lymphoblastic leukemia (ALL) occur during induction treatment. Many markers used for phenotyping and minimal residual disease (MRD) monitoring are affected. Glucocorticoid (GC)-induced expression modulation has been causally suspected, however, subclone selection may also cause the phenomenon.

Methods: We investigated this by following the phenotypic evolution of leukemic cells with flow cytometry from diagnosis to four time points during and after GC containing chemotherapy in the 20 (of 360 consecutive) B-cell precursor patients with ALL who had persistent MRD throughout.

Results: The early expression changes of CD10 and CD34 were reversible after stop of GC containing chemotherapy. Modulation of CD20 and CD45 occurred mostly during the GC phase, whereas CD11a also changed later on. Blast cells at diagnosis falling into gates designed according to "shifted" phenotypes from follow-up did not form clusters and were frequently less numerous than later on.

Conclusions: Our data support the idea that drug-induced modulation rather than selection causes the phenomenon. The good message for MRD assessment is that modulation is transient in at least two (CD10 and CD34) of the five prominent antigens investigated and reverts to initial aberrant patterns after stop of GC therapy, whereas CD20 expression gains new aberrations exploitable for MRD detection.
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Key terms: lymphoblastic leukemia; minimal residual disease; phenotype; antigen expression; flow cytometry

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Cells of acute lymphoblastic leukemia (ALL) differ from normal lymphoid precursors by mostly quantitative aberrations in their tissue- and differentiation-associated antigenic expression profile (1,2). Malignant immunophenotypes are considered to be constant phenomenon useful for leukemia subtype categorization, for anticipating genetic lesions and outcome, and, in particular, for detecting minimal residual disease (MRD) by flow cytometry (FCM) (3,4). Phenotypic changes, however, are sometimes recognized in recurrent disease and are frequently caused by clonal evolution and selection (3,5). For MRD assessment, such phenotypic shifts pose a particular challenge since they can cause misinterpretations and false negative results. Applying multiple marker combinations is considered an appropriate counter strategy (3).

During the course of a large multicenter study of MRD in childhood ALL, we observed antigenic expression shifts that frequently occurred already during the first weeks of treatment, both in CD10-positive B-cell precursor (BCP)-ALL and T-ALL (6,7). This phenomenon affects most markers broadly used as backbone in leukemia typing and current MRD-detection strategies, i.e., CD10, CD19, CD20, CD34, CD45, and TdT. This "new" type of expression changes could be due to selection of leukemic subclones, which harbor "shifted" antigenic profiles already at diagnosis and sustain the first period of chemotherapy better than the bulk of leukemia cells (8). Alternatively, it may just be related to drug-induced cell death, resulting in loss of membrane antigens or higher nonspecific staining (9,10). More recently, however, we showed that this type of modulation can be elicited also in vitro in viable (annexin V negative) leukemic cells by incubation with low concentrations of prednisone (11). In these experiments, lymphoblast viability was sustained by bone marrow (BM) stroma cells, which is essential for them (12,13). On the basis of these observations, we considered that a drug-induced regulatory modulation of the cellular machinery of antigen expression is the predominant cause of the phenomenon, rather than subclone selection or apoptosis-related artifacts. Corroborating this view, we show in this report that the observed immunophenotypic modulation is reversible after the end of glucocorticoid (GC) containing chemotherapy. We also show that cells with the "shifted" complex phenotypes found later on are not present at diagnosis at relevant frequencies, which further argues against subclone selection as an explanation.

MATERIALS AND METHODS

Patients, Samples, and Treatment

Four hundred four consecutive patients with ALL (age 1–18 years) were recruited in Monza ($n = 105$) and Vienna ($n = 299$) in a study of flow cytometric MRD assessment, which was conducted as a four-center collaboration. We focused on patients from the two centers because of technical similarities: usage of same cytometers, similar staining setup in terms of monoclonal antibody (MoAb) clones, fluorochromes, as well as panel combinations—see below. Only the 360 patients with a BCP-ALL (89% of all) were analyzed for this study. Peripheral blood (PB) and BM samples were received at diagnosis and from follow-up during induction treatment: PB from days 8 and 15, BM from days 15, 33, 52, and 78. MRD investigations were approved as part of the international trial by the institutional ethical committees and were strictly done according to informed consent guidelines. Induction treatment was performed according to trial AIEOP-BFM ALL 2000 (registered at http://www.clinicaltrials.gov/ as NCT00430118). It consisted of a 7-day prephase with daily oral prednisolone (60 mg/m^2 body surface area/day). From day 8 onward, patients received a randomized four-drug regimen until day 33 containing either prednisolone (60 mg/m^2 daily) or dexamethasone (10 mg/m^2 daily), plus L-asparaginase (8 × 5,000 U/m^2), daunorubicin (4 × 30 mg/m^2), and vincristine (4 × 1.5 mg/m^2). After day 28, GC medication was tapered over 9 days, and polychemotherapy was continued between day 36 until day 64 as extended induction with oral 6-mercaptopurine (60 mg/m^2 daily), cyclophosphamide (2 × 1000 mg/m^2), and low-dose cytarabine (16 × 75 mg/m^2). All patients also received intrathecal therapy with methotrexate (age adjusted) on days 1, 12, 33, 45, and 59.

Flow Cytometry

The standardization process including sample preparation, choice of MoAb clones, stain-lyse procedure, and flow cytometric analysis has been recently described in detail (14). MoAbs were assorted to fixed quadruple combinations: CD20/CD10/CD19/CD34, CD58/CD10/CD19/CD34, CD10/CD34/CD19/CD45, and CD10/CD11a/CD19/CD45 (ordered by channel 1–4: fluorescein isothiocyanate, FITC; phycoerythrin, PE; phycoerythrin-cyanin 7, PE-Cy7; allophycocyanin, APC). The instrument setup was optimized daily by analyzing

Table 1
Antigen Expression Levels (Median and Range of Geo-Mean MFI Values) of leukemic Blasts and Control Cells at Diagnosis and During Follow-Up Time Points

Antigen	*N*	d0	d15	d33	d52	d78
CD10	20	343 (55–642)	96 (9–430)	84 (10–231)	171 (14–449)	215 (29–912)
CD34	19	421 (3–1075)	61 (2–529)	194 (19–2483)	293 (11–5771)	375 (6–1734)
CD20	19	22 (2–526)	35 (2–895)	72 (3–312)	63 (4–982)	84 (5–879)
CD45	19	22 (2–319)	46 (4–461)	110 (3–400)	115 (3–502)	109 (2–419)
CD11a	18	60 (3–438)	46 (9–173)	28 (2–130)	82 (3–363)	149 (4–522)
Control[a]	17	650 (216–917)	489 (219–942)	567 (242–993)	643 (203–1501)	700 (278–1075)

[a]Control, i.e., CD45 expression on normal mature B cells from within the samples.

Calibrite™ beads (BD) and normal adult PB cells stained with CD4/CD8/CD3/CD45 (4).

Immunophenotyping at diagnosis and quality control evaluations were performed collecting at least 30,000 cellular events, while for MRD measurements 300,000 events were acquired per MoAb combination from 700,000 stained cells. Cell acquisition was performed with FACSCalibur™ cytometers (BD) using the CELL Quest™ software (BD). Data analysis was done with the PAINT-A-GATE™ software (BD). Leukemic cells were identified using an immunological gate (associated with 90° scattering, SSC), which included all CD19 positive cells. MRD was defined as an accumulation of at least 10 clustered events displaying lymphoid scattering properties and leukemia-associated immunophenotypic characteristics as reported (2,15,16). Skewing of the proportional MRD-quantification by irrelevant non-nucleated events, like erythrocytes, platelets, and debris, was avoided by relating MRD only to SYTO®16-positive, i.e., nucleated and cellular events (15). Absolute MRD values (blasts per microliter) were calculated using the cell count of the sample (assessed with a conventional hemocytometer) and the relative MRD estimate from FCM analysis (15).

Antigen expression was quantified on the basis of mean fluorescence intensity (MFI) values as reported using the CELL Quest™ software (7,11). DAKO Fluorospheres™ (type IIIa beads) with assigned values of molecules of equivalent soluble fluorochrome were used for intracenter longitudinal monitoring of instrument performance stability (17). One hundred thirty-seven measurements of these beads (which contain blank as well as quadruple-fluorescence beads with five different emission intensities) were performed during the study period. These measurements gave standard deviations of mean fluorescence emissions of ±12.4–14.9% (min–max) in the FITC channel, of ±10.1–12.2% in the PE channel, and of ±19.3–24.1% in the APC channel. These proportions reflect the low background of time-related variance of measured MFI values in our study.

Statistical Analyses

The Wilcoxon-signed rank test was used to longitudinally compare the median MFI values of samples from diagnosis and follow-up per patient. Box and whisker plots were used for descriptive purposes.

RESULTS AND DISCUSSION

Evolution of Antigen Expression During and After GC Containing Chemotherapy

Among the 360 patients, we found 20 (5.6%) with a CD10-positive BCP-ALL to be MRD-positive in BM throughout the total evaluation period until including day 78. The mean proportion of MRD in BM, which was investigated in the latter patients per time point, was 60.4% at diagnosis (±28.3% standard deviation), 22.2% (±21.5) at day 15, 6.4% (±11.9) at day 33, 1.8% (±4.4) at day 52, and 0.37% (±0.71) at day 78. The evolution of antigen expression over time is summarized in Table 1 as well as in Figure 1. Importantly, these analyses were done by strictly gating on intact cellular events with lymphoid scattering properties (18). CD10 and CD34 expression significantly declined until day 33 and recovered thereafter till day 78 to values comparable with those at diagnosis. Notably, CD34 expression was highly variable during follow-up and tended to reincrease in some patients already at day 33, a few days after start of tapering of GCs. This pattern of decrease and reincrease favorably matches with the schedule of GC containing chemotherapy as well as with the direction of the in vitro GC-induced expression alterations of these two antigens (11). It may therefore be regarded a transient, GC-induced phenomenon related to modulation of responsive genes and of the translational machinery, as discussed recently (19,20). CD20 and CD45 expression increased between diagnosis and follow-up, but changes between days 15/33 and later time points were not significant. CD11a expression showed a high degree of interpatient variability both already at diagnosis and also later on. An increase in its expression was significant in comparisons between diagnosis and both days 15 and 78, respectively. Since the expression of these three latter antigens did not "normalize" to patterns found at diagnosis, the modulation induced by GC may not be the only precipitating factor involved. An example of the shifts of antigen expression over time in an individual patient is shown in Figure 2.

Quantification of MRD with "Shifted" Phenotypes Within the First 2 Weeks of GC Containing Therapy

Selection of subclones with phenotypes different from the bulk of blast cells at diagnosis may also serve as an explanation for the phenomenon of expression

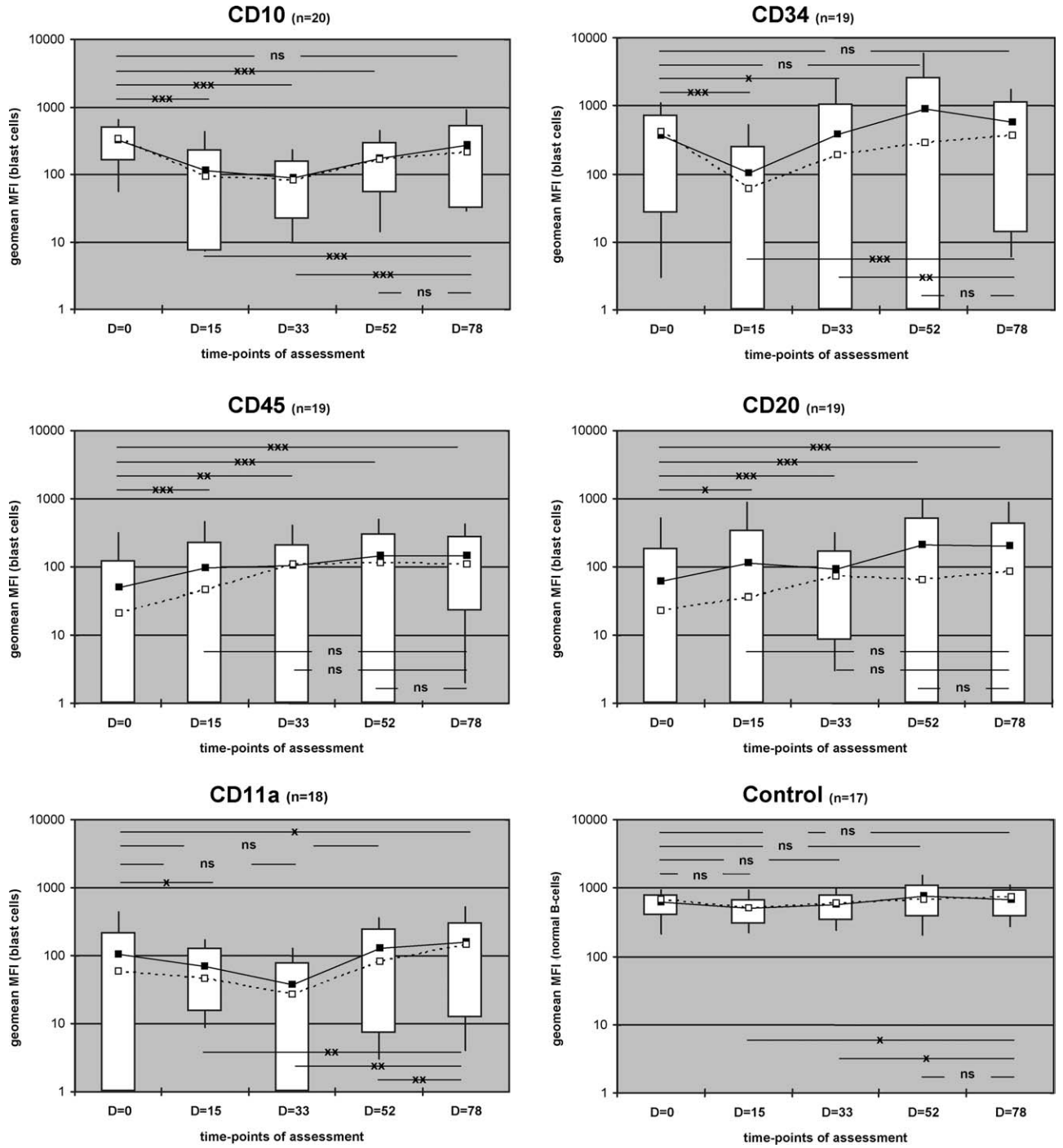

FIG. 1. Evolution of expression of five antigens relevant to MRD studies on leukemic blasts from patients with detectable MRD during (days 15 and 33) and after GC containing polychemotherapy (days 52 and 78). Day = 0 represents data from diagnosis. As control, CD45 expression of normal residual mature B cells from within the samples from diagnosis or follow-up was also analyzed. Antigen expression of the investigated cell populations is represented by geo-mean MFI values. Boxes (representing the standard deviation; mind the logarithmic scale) and whiskers (minimum–maximum) summarize the distribution of data. Mean and median values of the cohorts are represented by solid and open data points, respectively, and the accompanying solid and dashed lines reflect their evolution. Statistical analyses of paired data from time point combinations are reflected by horizontal lines with symbols (ns, not significant; x, $P \leq 0.05$; xx, $P \leq 0.02$; xxx, $P < 0.01$).

modulation during induction therapy (8). To investigate whether this explanation was plausible, we quantified and compared blasts with "shifted" phenotypes found at early follow-up time points (days 8 and 15) with those bearing similar characteristics already at diagnosis of BCP-ALL. We used only PB samples (to avoid any bias by hemodilution of BM) and quantified absolute MRD values as blast cells per microliter of sample. Out of a

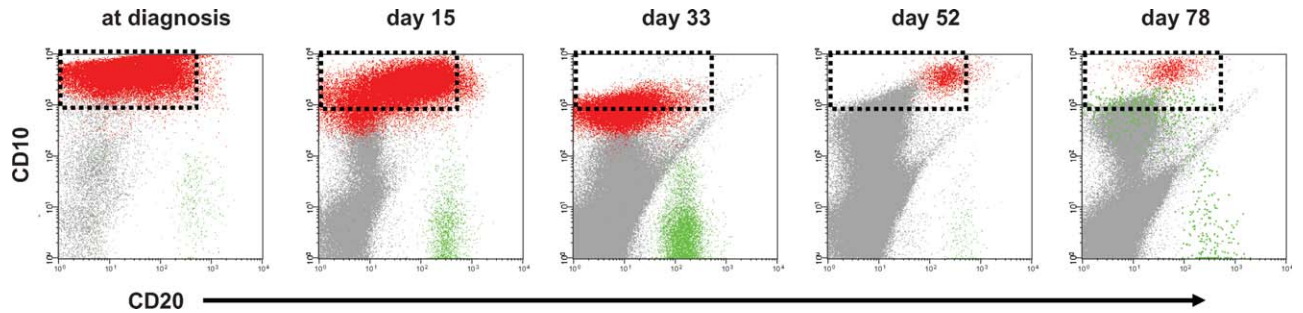

FIG. 2. Down modulation of CD10 is transitory and dependent on the phase of treatment. Dot-plots of four-color flow cytometric analyses of BM samples from diagnosis and follow-up time points of a patient during (days 15 and 33) and after GC containing polychemotherapy (days 52 and 78) are shown. Leukemic cells appear in red, normal B-lymphocytes in green, and all other cells are gray. Note that during GC containing treatment expression of CD10 decreases on leukemic cells, but reincreases thereafter to levels found at diagnosis. Further note that normal B cells at days 15, 33, and 52 exclusively exhibit the $CD20^{++}CD10^{+/-}$ phenotype of stage 3 mature cells, whereas at day 78 also regenerating normal B-cell precursors with strong CD10 expression but low CD20 are visible (stage 1; for stage definition see Ref. 21). The latter would not be distinguishable from leukemic blasts when occurring at day 33 but at day 78 are clearly distinct from MRD cells which in the meantime also acquired CD20 expression.

consecutive series of 39 patients screened, we could assess data of 12 patients because they fulfilled three criteria for further analysis: (i) clearly obvious expression modulation at follow-up by visual dot plot inspection, (ii) more than one antigen involved, and (iii) MRD-positive follow-up samples. Using a back-gating strategy with

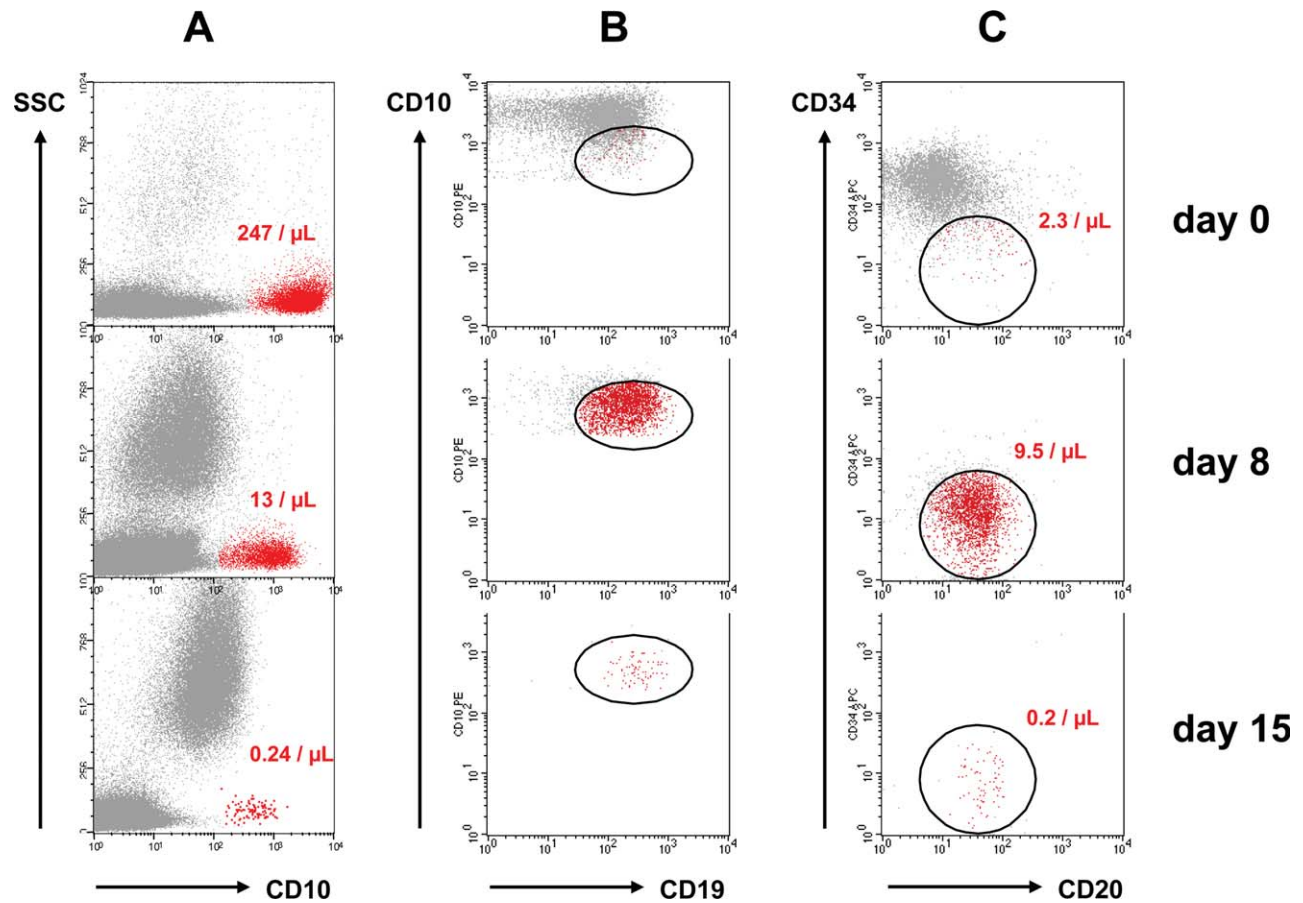

FIG. 3. Leukemic cells with modulated phenotypes do not form subpopulations at diagnosis and are frequently less numerous than blasts with similar phenotypic characteristics in early follow-up. Peripheral blood of a case of BCP-ALL was analyzed with CD20/CD10/CD19/CD34 in four-color FCM at diagnosis (upper row), at day 8 (center), and at day 15 (lower). In (**A**), ungated dot-plot analyses are shown. All leukemic blasts, characterized by CD10 expression, are red, whereas other cells are gray. In (**B**) (CD10 vs. CD19) and in (**C**) (CD34 vs. CD20), only gated $CD10^{+}$ blasts are shown. Gates were set according to the blast cell phenotype at day 15, and identical gates were introduced into dot plots from diagnosis and day 8. Blasts that fall into both gates in combination are depicted in red. At diagnosis, the few blasts that bear all modulated phenotypic features are dispersedly located in the gates, different from the respective blasts at days 8 and 15, which clearly form populations. Note the absolute blast counts [in (A), total blasts; in (C) blasts that bear all modulated phenotypic features] at the sequential time points.

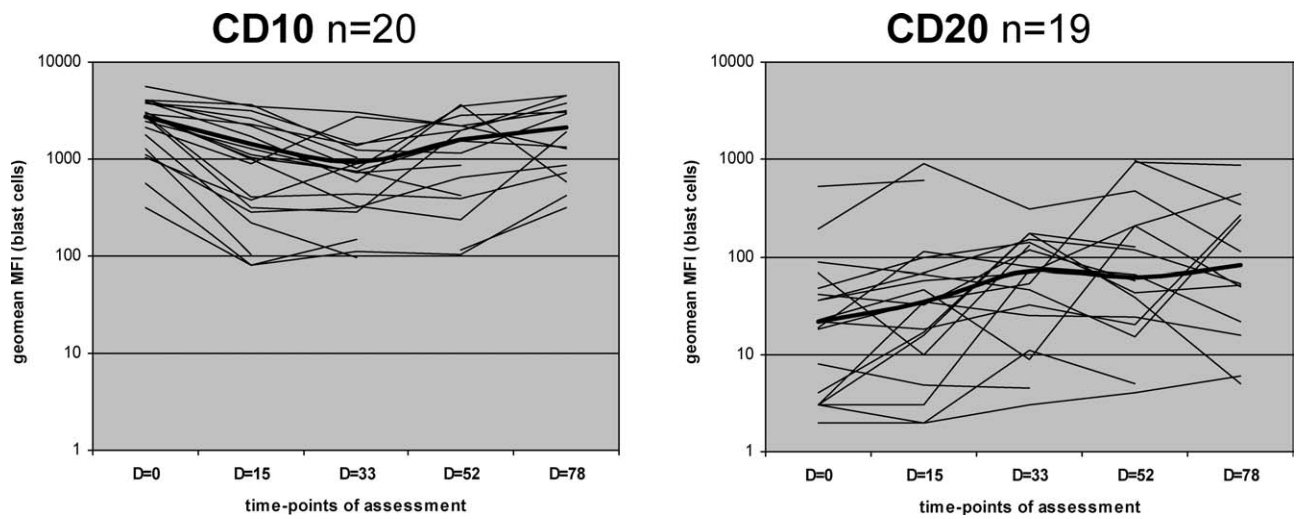

FIG. 4. Intrapatient evolution of expression of CD10 and CD20 on leukemic blasts from patients with detectable MRD during (days 15 and 33) and after GC containing polychemotherapy (days 52 and 78). Antigen expression of the investigated cell populations is represented by geo-mean MFI values. Median values of the cohorts are represented by bold lines. Note that while evolution of CD10 follows rather uniform patterns, CD20 patterns are more variable with a trend to gradual upregulation.

fixed gates set according to "shifted" blast immunophenotypes at follow-up, we found that leukemic cells at diagnosis, which fell into these gates, did not clearly form subpopulations distinct from the majority of leukemia in any of the 12 analyzed cases. Rather, we found at diagnosis cells with "shifted" phenotypes (in median 1.1% of all PB blasts, range 47% to 0.07%) dispersed in the marginal zones of general populations (Fig. 3). In absolute values, at diagnosis the bulk of leukemia counted in median 5,091 cells/μL (range 102–50,452), and the subset falling into the gate (shifted phenotype) 52 cells/μL (1.3–9,180). In follow-up, blasts within the gate counted in median 23.5 cells per microliter (1–231, day 8). These were mostly representative of the majority of residual leukemia in follow-up (day 8: 76% in median [range 12–100%]; day 15 ($n = 5$): 67% [39–100%]). Blasts with "shifted" phenotypes were even more numerous on day 8 compared with diagnosis in 5 of the 12 paired samples (increase factor: 2.0 in median; range 28.6–1.1), whereas the total blast count decreased in these samples in median 19-fold (7.3–212) on effective treatment. Even at day 15, there was one patient out of five with MRD where blast cells with "shifted" phenotype were 3.6-times more numerous than those at diagnosis with similar characteristics. None of the evaluated patients relapsed during the follow-up period of 4 years and 9 months in median. Taken together, blasts with "shifted" immunophenotypes (i) did not form distinct clusters at diagnosis and (ii) were frequently less numerous at diagnosis when compared with follow-up (Fig. 3). These findings argue against subclone selection as the predominant mechanism underlying the observed phenotypic changes during early treatment. Rather, drug-induced modulation of antigen expression seems to be a more plausible mechanism leading to relocation of a majority of blasts at follow-up time points to different dot-plot regions compared with those of initial leukemia. If so, phenotypic changes obvious as early as on day 8 of treatment with BFM-type protocols must be inducible by therapy only with daily prednisone and one initial intrathecal injection of methotrexate. This has recently been suggested by studies on the RNA level (19).

In conclusion, we consider our data as further indirect evidence for drug-induced modulation rather than selection as the more general mechanism underlying the phenomenon of antigen expression changes during the early treatment phases of ALL. Nevertheless, we are aware that in individual cases selection or cell death-related artifacts may also occur. Hence, careful application of multiparameter FCM is necessary to warrant correct and reliable MRD results in leukemia follow-up. This must include not only usage of multiple markers at a time to exclude false negativity due to phenotypic changes. It must also include knowledge on the typical background and the phenotypic patterns of normal cells at distinct time points of follow-up, as well as on the leukemia-inherent potentialities to early phenotypic changes. The latter follow generic patterns, but occur with some interindividual variability (7). In this respect, we have recently shown that the amount of phenotypic shifts correlates positively with treatment sensitivity in ALL (11). Nevertheless, the good message for MRD studies is that phenotypic shifts of highly relevant antigens like CD10 and CD34 are only transitory and revert to initial aberrant patterns after stop of GC containing therapy in many patients (Fig. 4). In addition, the phenomenon of phenotypic modulation tends to create even new combinations of aberrant expressions during follow-up (Fig. 2), in particular based on upregulation of CD20 (Fig. 4). With respect to the latter phenomenon, we have evidence that this occurs in about 30–50% of patients with BCP-ALL during induction (22). Together

with reverting aberrations in CD10 or CD34 expression this effect adds favorably to the potentialities of FCM in assessing MRD in ALL.

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